APPLICATION FOR UNITED STATES PATENT

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Title: PRODUCTION OF VACCINIA VIRUS RECOMBINANTS THAT EXPRESS HEPATITIS B VIRUS SURFACE ANTIGEN

Abstract of the Disclosure

Methods and compositions are provided for the construction and use of infectious vaccinia virus recombinants that contain the hepatitis B virus surface antigen (HBsAg) gene linked to a vaccinia virus promoter. Cells infected with the vaccinia virus recombinants synthesize and excrete HBsAg. Rabbits inoculated with this vaccine produce antibodies to HBsAg.

Background of Invention

This application is a continuation in part of our application filed November 30, 1982, entitled "Process for Using Poxviruses as Vectors for Expression of Foreign Genes." (Serial No. 445,451)

Improved procedures are needed to prevent and treat disease caused by hepatitis B virus. It has been estimated that nearly 200 million people are chronically infected with this virus and large numbers of deaths are attributed to sequelae of hepatitis. Although repeated immunizations with HBsAg prevent primary infections, vaccines prepared from the blood of human hepatitis B virus carriers is expensive and available in limited supply. The production of hepatitis B virus surface antigen from other sources might alleviate part of the problem. In this regard, the hepatitis B virus genome has been cloned in phage λ and plasmid vectors and its primary structure has been determined. A continuous nucleotide sequence that could encode the HBsAg was identified and expression was obtained in Escherichia coli, yeast and cells infected with defective SV40 virus recombinants. Nevertheless, HBsAg from such sources would require extensive purification if used for medical purposes. A live virus vaccine might provide a useful alternative to a subunit vaccine. However, the absence of attenuated hepatitis B virus strains and the inability to grow this virus in cell culture heretofore prevented such an approach.

Vaccinia virus has been effectively used for immunization against a related virus that causes smallpox. Recently, a general process has been developed for the molecular cloning and expression of foreign genes in a vaccinia virus vector (Moss, Mackett, and Smith, U. S. Patent Application - Process for Using Poxviruses as Vectors for Expression of Foreign Genes, filed November 30, 1982; adjective material of this prior application is hereby incorporated by reference). The method depends on

the formation of chimeric genes consisting of vaccinia virus transcriptional regulatory sequences ligated to foreign protein coding sequences in vitro and the insertion of these chimeric genes into the vaccinia virus genome by homologous recombination in vivo. Using this procedure, infectious vaccinia virus recombinants that express genes of other DNA viruses, RNA viruses, and prokaryotes have been obtained. We considered that this process could provide the basis for developing live vaccines against diseases of man and animals. This disclosure provides methods and compositions for the production of a vaccinia virus recombinant that expressed HBsAg upon infection of cells or animals.

Prior Art Statement

References which relate to the subject invention are: Mackett et al., Proc. Natl. Acad. Sci. U.S.A., in press; Moss, Mackett and Smith, U. S. Patent Application, Process for Using Poxviruses as Vectors for Expression of Foreign Genes, submitted 11/30/82; Panicalli and Paoletti, Proc. Natl. Acad. Sci. U.S.A. 79:4927-4931 (1982); Valuenzuela et al., Nature 298:347-350 (1982); Moriarity et al., Proc. Natl. Acad. Sci. U.S.A. 78:2606-2610 (1981); Liu et al., DNA 1:213-221 (1982).

Utility Statement

Methods and compositions are provided for the construction and use of vaccinia virus recombinants that contain the HBsAg gene under control of vaccinia virus promoters. Cells infected with these vaccinia virus recombinants synthesize and excrete HBsAg. The ability of the recombinant virus to serve as a vaccine was demonstrated by intradermal inoculation of rabbits. Following vaccination, the rabbits produced antibodies to HBsAg as measured by standard immunological tests.

Summary of Invention

Chimeric genes, consisting of a vaccinia virus transcriptional regulatory sequence ligated to an HBsAg coding sequence, flanked by vaccinia virus DNA, were constructed using plasmids specifically designed for this purpose. Plasmids containing the chimeric genes were introduced by transfection into cells infected with vaccinia virus where homologous recombination occurred. Vaccinia virus recombinants were isolated by selective methods and shown to contain the HBsAg gene. Cells infected with the vaccinia virus recombinants synthesized and excreted HBsAg. Rabbits successfully vaccinated with the virus recombinants produced antibodies to HBsAg.

Description of Invention

1. Use of specially constructed plasmid vectors (pGS20, pGS21, and pMM3) to form a chimeric gene, consisting of vaccinia virus transcriptional regulatory sequences ligated to HBsAg coding sequences, flanked by vaccinia virus DNA.

Several plasmid vectors, including pGS20, pGS21, and pMM3 were constructed to be generally usable for the molecular cloning and expression of foreign genes in vaccinia virus (Moss, Mackett and Smith, U.S. Patent Application - Process for Using Poxviruses as Vectors for Expression of Foreign Genes, submitted 11/30/82). Two of these plasmids, pGS20 and pGS21, have a vaccinia virus promoter, translocated from a gene that encodes the 7.5K polypeptide, linked to multiple restriction endonuclease sites and inserted into the coding sequence of the vaccinia virus thymidine kinase (TK) gene. The other plasmid, pMM3, has restriction endonuclease sites engineered next to the vaccinia virus TK promoter. Each of the plasmids were designed so that restriction

endonuclease sites would be available for any foreign protein coding sequence to be inserted next to a vaccinia virus promoter. In addition, the interruption of the vaccinia virus TK gene led to a simple method of selecting recombinant vaccinia virus.

A 1,350 bp DNA fragment containing the HBsAg gene was obtained from a plasmid [Moriarity et al., Proc. Natl. Acad. Sci. U.S.A. 78:2606-2670 (1981)]. Nucleotide sequence data suggested that the first ATG codon after one of the BamHI restriction endonuclease sites represented the initial methionine residue of HBsAg. The fragment containing the HBsAg gene was isolated from 50 µg of this plasmid by digestion with 100 units of BamHI in 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 10 mM dithiothrietol (DTT) (hereafter called medium restriction buffer) for 2 hr at 37°C. DNA fragments were separated by electrophoresis at 200 volts for 1 hr through a 1% agarose gel containing 40 mM Tris-acetate (Ac) (pH 8.0), 20 mM NaAc, 2 mM EDTA, 18 mM NaCl. The gel was soaked in 1 µg/ml ethidium bromide (EtBr) and DNA fragments were visualized by illumination with long wave ultraviolet light. A gel strip containing a DNA fragment of 1.35 kilobase pairs (kb) was excised from the agarose gel and DNA within this strip was transferred to diethylaminoethyl (DEAE)-cellulose by electroblotting in 40 mM Tris-Ac (pH 7.2), 20 mM NaAc, 1 mM EDTA for 1 hr at 2.5 mA. DNA was eluted from the DEAE-cellulose paper by shaking in 1.2 M NaCl, 40 mM Tris-Ac (pH 7.2), 20 mM NaAc, 1 mM EDTA for 30 min at 25°C and recovered from the supernatant by addition of 2 volumes of ethanol followed by centrifugation at 12,000 X g for 5 min.

The plasmids pGS20, pGS21, and pMM3 were linearized by digestion with 2 units of BamHI/µg DNA in medium salt restriction buffer for 2 hr at 37°C. The linearized plasmids were then dephosphorylated at their 5' termini by incubation with 0.1 unit of calf intestinal alkaline phosphatase in 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine for 30 min at 37°C. After two extractions with equal volumes

of phenol:chloroform (1:1), the DNA was recovered by ethanol precipitation and centrifugation. 0.5 μg of each linearized, dephosphorylated plasmid was ligated with 0.2 μg of the 1.35 kb BamHI fragment containing the HBsAg gene in 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP for 15 hr at 12°C. Ligated DNA was used to transform competent E. coli cells strain HB101 and the transformed cells were grown for 15 hr at 37°C on L-broth plates containing 1.5% bacto-agar and 50 μg/ml ampicillin. Bacterial colonies were picked and grown in 10 ml of L-broth containing 50 μg/ml of ampicillin for 15 hr at 37°C. Plasmid DNA was prepared from 1.5 ml of bacterial cultures by the following mini-preparation of plasmid DNA. Bacterial cells were pelleted by centrifugation (12,000 X g, 1 min) and resuspended in 0.1 ml of lysis solution [25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose, 2 μg/ml lysozyme] and incubated on ice for 30 min. 0.2 ml of 0.2 M NaOH, 1% sodium dodecyl sulfate (SDS) was added and the mixture incubated on ice for 5 min. 0.15 ml of 3M NaAc (pH 4.8) was added and after a further 1 hr incubation on ice, the mixture was centrifuged at 12,000 X g for 5 min. Plasmid DNA was precipitated from the supernatant by addition of 1 ml of ethanol, recovered by centrifugation (12,000 X g, 5 min) and finally redissolved in 0.1 ml of 10 mM Tris-HCl (pH 8.0) 1 mM EDTA (TE buffer).

Plasmid DNAs were screened for the presence of the 1.35 kb BamHI fragment containing the HBsAg gene by digestion of 10% of each plasmid DNA sample with 5 units of restriction endonuclease BamHI in medium salt restriction buffer for 1 hr at 37°C, followed by agarose gel electrophoresis and EtBr staining.

Since the 1.35 kb BamHI hepatitis B virus DNA fragment could be inserted in either of two orientations within each of the plasmids, additional screening was necessary. Plasmids derived from pGS20 and pGS21 were digested with XbaI restriction endonuclease in 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$ (high salt restriction buffer) for

1 hr at 37°C and analyzed by agarose gel electrophoresis. The identification of XbaI fragments of approximately 830 or 1,730 bp discriminated betwen derivatives of pGS20 that contained the HBsAg gene in incorrect and correct orientations, respectively. XbaI fragments of 2,150 bp or 1,150 bp discriminated between derivatives of pGS21 that had the HBsAg gene in incorrect and correct orientations, respectively. Plasmids derived from pMM3 were screened by HincII digestion in medium salt restriction buffer followed by agarose gel electrophoresis. The presence of the HBsAg gene in correct orientation was indicated by generation of fragments of approximately 5,400 bp and 200 bp whereas fragments of 4,400 bp and 1,200 bp resulted when HBsAg was in the incorrect orientation. Plasmids were then grown, amplified and purified as follows. Transformed bacteria were seeded into 400 ml cultures of M-9 medium containing 150 µg/ml leucine, 150 µg/ml proline, 0.8 µg/ml vitamin B_1, 50 µg/ml ampicillin and grown at 37°C until the culture reached an optical density of 0.8 at 590 nm. Chloramphenicol was then added to a concentration of 200 µg/ml and the culture was incubated for 12 hr at 37°C. Bacteria were pelleted by centrifugation (5,000 X g, 10 min), washed once in 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5) and then resuspended in 10 ml of lysis solution (above). After incubation for 30 min on ice, 20 ml of 0.2 M NaOH, 1% SDS was added and incubation continued for 5 min. 15 ml of 3 M NaAc (pH 4.8) was then added and after incubation on ice for a further 1 hr, the mixture was centrifuged at 10,000 X g for 10 min. The supernatant was recentrifuged at 10,000 X g for 10 min and plasmid DNA was precipitated from the final supernatant by addition of 2 volumes of ethanol. After centrifugation at 10,000 X g for 5 min, the pellet was redissolved in 10 ml of TE buffer and the solution was extracted twice with equal volumes of phenol:chloroform. DNA was recovered by ethanol precipitation and centrifugation and redissovled in 5 ml of TE buffer. 0.1 mg/ml of ribonuclease (heated at 100°C to inactivate contaminating deoxyribonucleases) was added and the mixture

was incubated for 30 min at 37°C. DNA was then precipitated by addition of NaAc (pH 7) to 0.1 M and 1.5 volumes of ethanol and was recovered by centrifugation at 5,000 X g for 5 min. Remaining RNA was removed from the DNA by dissolving the pellet in 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and passage through a sephacryl-S300 column equilibrated with the same buffer. DNA eluting in the first peak was recovered by ethanol precipitation and centrifugation, dissolved in 1.0 ml of TE buffer and stored at 4°C. Plasmids from pGS21 that have the HBsAg gene in incorrect and correct orientations relative to the translocated promoter have been designated pHBs1 and pHBs2, respectively. Plasmids from pGS20 that have the HBsAg gene in incorrect and correct orientations have been designated pHBs3 and pHBs4. The plasmid from pMM3 that has the HBsAg gene in correct orientation relative to the TK promoter has been designated pHBs5.

2. Formation of vaccinia virus recombinants containing a chimeric HBsAg gene.

Plasmids containing a chimeric HBsAg gene flanked by segments of the vaccinia virus TK gene were used to transfect cells infected with wild-type thymidine kinase positive (TK^+) vaccinia virus. Confluent monolayers of CV-1 or MRC-5 cells (25 sq cm) were infected with vaccinia virus at 0.01 plaque forming units (pfu) per cell. One µg of plasmid, 1 µg of vaccinia virus DNA and 20 µg of calf thymus DNA were mixed in 1 ml of 0.1% dextrose, 0.14 M NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 20 mM Hepes and precipitated by addition of $CaCl_2$ to a final concentration of 0.125 M. The mixture was agitated gently for 45 min at 25°C and 0.8 ml of the fine suspension was added at 2 hr after infection to a monolayer from which medium had been removed. After 30 min at 25°C, 7.2 ml of Eagle medium containing 8% fetal bovine serum (FBS) was added and the incubation was continued at 37°C for a further 3.5 hr. At 6 hr after infection, the medium was replaced with fresh medium containing 8% FBS

and the incubation was continued for 2 days. Cells were then scraped from the bottle, pelleted, resuspended in 0.5 ml of tissue culture medium and homogenized to break cells and liberate the virus.

Only a small percentage of the virus produced in transfected cells was recombinant. These recombinants were isolated by selective and non-selective methods. Selection was possible because the HBsAg coding sequences were inserted into the TK gene and interrupted its function. Because of the thymidine kinase negative (TK^-) phenotype, recombinant virus was able to form plaques in the presence of 5-bromodeoxyuridine (BUdR) whereas the original TK^+ virus did not. Non-selective procedures were carried out as described by Villarreal and Berg [Science 196:183-185 (1977)].

Monolayers of TK^-143 cells were inoculated with virus from transfected cells and 2 hr later were overlayed with medium containing 1% low melting agarose, 25 μg/ml BUdR, and 2.5% FBS. After 3 days at 37°C in a humidified 5% CO_2 atmosphere, the cells were stained with 0.005% neutral red. Greater than 50% of the plaques visualized under these conditions were shown by dot blot hybridization to contain recombinants with hepatitis B virus DNA. For the latter procedure, individual virus plaques were picked using a sterile Pasteur pipette and used to infect TK^-143 cell monolayers in 16 mm diameter multiwell dishes. After 48 hr of incubation in medium containing BUdR at 37°C, the cells were scraped, lysed by three freeze-thaw cycles, and collected on nitrocellulose sheets by filtration through a micro-sample manifold (Schleicher and Schuell, NH). The filter was washed with 100 mM NaCl, 50 mM Tris-HCl, pH 7.5), blotted 3 times on successive Whatman 3 MM filter papers saturated with (1) 0.5 M NaOH, (2) 1 M Tris-HCl (pH 7.5), and (3) 2 X SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), baked at 80°C for 2 hr and then incubated with 5 X Denhardt's solution [Denhardt, Biochem. Biophys. Res. Commun. 23:641-646 (1966)] supplemented with 0.1 mg/ml of denatured, sheared salmon sperm DNA in 4 X SSC at 65°C for 4 hr. Hepatitis B virus DNA,

labeled with ^{32}P by nick translation [Rigby et al., J. Mol. Biol. 113, 237 (1975)], and sodium dodecyl sulfate (SDS) at a final concentration of 0.1% were added and hybridization continued for 15 hr at 65°C. The filter was washed twice for 15 min at 65°C with 2 X SSC/0.1% SDS and then with 0.2 X SSC/0.1% SDS. An autoradiograph was made by placing the filter next to X-ray film and the presence of dark spots on developed film identified recombinant virus containing the HBsAg gene. Recombinant virus was plaque purified a second time in TK^-143 cells with an agar overlay containing 25 μg/ml of BUdR and recombinant virus stocks were then prepared. The recombinant virus stocks were designated vHBs1, vHBs2, vHBs3, vHBs4 and vHBs5 to correspond with the names given to the plasmids from which they were derived.

3. Expression of HBsAg in cells infected with vaccinia virus recombinants.

Evidence for HBsAg expression in cells infected with vaccinia virus recombinants was obtained by a standard radioimmunoassay procedure (AUSRIA, Abbot Laboratories). Monolayers of CV-1 cells were infected with 10 plaque forming units (pfu) per cell of vaccinia virus recombinants, wild-type vaccinia virus, or mock infected as indicated in Table 1. Two hr after infection, the virus inoculum was removed and cells were overlayed with Eagles medium containing 2.5% FBS. After incubation for 24 hr, the cells were scraped from culture flasks and separated from culture medium by centrifugation (2,000 X g, 5 min). Cell pellets were resuspended in 0.5 ml of TBS [0.15 M NaCl, 10 mM Tris-HCl (pH 7.5)], frozen and thawed 3 times, sonicated and centrifuged (12,000 X g, 1 min) to remove debris. The supernatant and tissue culture medium were assayed for HBsAg by radioimmunoassay. Beads coated with anti-HBsAg antibody were incubated with 0.2 ml of sample for 2 hr at 45°C, thoroughly rinsed 8 times with 1 ml H_2O and then reincubated with ^{125}I-labeled antibody against HBsAg for 1 hr at 45°C. Beads were washed again and then counted

in a gamma-ray scintillation counter. The quantity of HBsAg present was calculated by reference to positive and negative controls supplied by Abbot Laboratories and incubated in parallel.

As shown in Table 1, HBsAg was produced in greatest amount by cells infected with vHBs2 and vHBs4. Lesser amounts of HBsAg were made in cells infected with vHBs5. By contrast, those recombinants that did not have the HBsAg gene in correct orientation with the translocated vaccinia virus promoter, vHBs1 and vHBs3, made barely detectable levels of HBsAg. Significantly, much of the HBsAg was excreted into the culture medium. Release of HBsAg was not due to cell lysis since 90% of infectious virus remained cell associated. Table 1 also shows that the similar yields of virus were obtained after infection with wild-type (WT) or recombinant virus.

The nature of the HBsAg synthesized by cells infected with vaccinia virus recombinants was analyzed by immunoprecipitation. Monolayers of CV-1 cells infected with purified vaccinia virus recombinants at 30 pfu/cell were incubated in Eagles medium containing 0.01 mM methionine for 4 hr after infection. The cells were then incubated for 20 min at 37°C with fresh Eagles medium without methionine supplemented with [^{35}S]methionine (120 µCi/5 X 10^6 cells). Excess [^{35}S]methionine was removed by washing the cells 3 times with ice-cold phosphate buffered saline and cell extracts were prepared by incubation of cells in 0.5 ml of 0.1% aprotinin, 0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.5% NP40, for 10 min on ice, followed by centrifugation. 80% of the supernatant was incubated with 25 µl of guinea pig non-immune serum at 4°C for 15 hr and immune complexes were removed by addition of 50 µl of a formalin-treated staphylococcal A suspension and incubation for 30 min at 4°C, followed by centrifugation. 20 µl of guinea pig HBsAg antiserum was then added, incubated for 15 hr at 4°C and then immune complexes were removed by addition of staphylococcal A suspension as above, followed by centrifugation. The pellet was washed twice in 0.05 M Tris-HCl (pH 7.5),

0.15 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and twice in 0.4M LiCl, 2M urea, 10 mM Tris-HCl (pH 8.0). Immune complexes were eluted from the staphylococcal A pellet by incubation in 50 µl of 0.06 M Tris-HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue for 15 min at 25°C. After centrifugation, the supernatant was boiled and electrophoresed through a 15% polyacrylamide gel. The gel was fixed, treated with Enhance (New England Nuclear Corporation) and a fluorograph was obtained. Examination of the fluorograph indicated that two polypeptides were specifically immunoprecipitated. These had molecular weights of 23,000 and 25,400 and comigrated on polyacrylamide gels with polypeptides immunoprecipitated by HBsAg antiserum from a hepatoma cell line PLC/PRF/5. The nature of the HBsAg excreted from cells infected with vaccinia virus recombinants was further examined. Tissue culture medium, harvested 24 hr after infection of CV-1 cells with 30 pfu/cell of vaccinia virus recombinant vHBs4, was clarified by centrifugation at 2,000 X g for 5 min and then recentrifuged at 75,000 X g for 24 hr at 4°C. The pellet was resuspended in 4.5 ml of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and then 1.38 grams of CsCl was added to a final density of 1.2 g/cm^3. The sample was centrifuged at 220,000 X g for 64 hr at 4°C and gradient fractions were collected. Diluted samples were tested for HBsAg by radioimmunoassay. A peak of HBsAg was detected at a density of 1.2 g/cm^3 which was identical to a peak of HBsAg obtained when culture medium from hepatoma cell line PLC/PRF/5 was treated in parallel. The peak fractions of HBsAg were dialyzed against phosphate buffered saline (PBS) and recentrifuged on a 5 to 30% (w/w) sucrose gradient in PBS for 4.5 hr at 150,000 X g at 4°C. Gradient fractions were collected and samples were diluted and assayed for HBsAg by radioimmunoassay. A peak of HBsAg was detected which sedimented at the same rate as the HBsAg from hepatoma cell line PLC/PRF/5. When samples from these peaks were analyzed by electron microscopy, particles of HBsAg were detected.

In all respects examined (including antigenicity, polypeptide composition, buoyant density, sedimentation rate), the HBsAg excreted from cells infected with vaccinia virus recombinant vHBs4 was indistinguishable from HBsAg particles released from hepatoma cell line PLC/PRF/5.

4. Vaccination of rabbits with vaccinia virus recombinants.

Previous studies have shown that HBsAg particles from the blood of human hepatitis B virus carriers are highly immunogenic and can neutralize the infectivity of hepatitis B virus. Consequently, the question of whether infection of animals with vaccinia virus recombinants that express HBsAg would induce production of anti-HBsAg antibodies was examined.

Rabbits were pre-bled and then infected with either wild-type vaccinia virus or vaccinia virus recombinant vHBs4 by intradermal injection of 10^8 pfu of virus into 4 sites on the back of each rabbit. Rabbits were bled daily following inoculation and serum was prepared and stored frozen at -70°C. At 5 days, the rabbits developed lesions at the sites of inoculation and by 10 days these lesions were visibly healing. Serum from the rabbits was tested for HBsAg and antibodies against HBsAg by radioimmunoassay, and for vaccinia virus by plaque assay. No HBsAg or vaccinia virus was detectable in the serum. However, by 5 days after inoculation, antibodies against HBsAg were detected (Table 2).

TABLE 1

Production of HBsAg and Vaccinia Virus from Infected Cells

	ng HBsAg/5 X 10^6 cells		Virus yield (pfu)	
	cell extract	culture medium	cell extract	culture medium
Uninfected	<1	<1	ND	ND
WT	<1	<1	7.8 X 10^8	7.7 X 10^7
vHBs1	11	20	8.3 X 10^8	10.2 X 10^7
vHBs2	835	1700	7.9 X 10^8	9.9 X 10^7
vHBs3	14	25	9.1 X 108	9.0 X 10^7
vHBs4	930	1700	8.8 X 10^8	9.8 X 10^7
vHBs5	35	80	10.3 X 10^8	9.6 X 10^7
Hepatoma cells	340	900	ND	ND

CV-1 cell monolayers were infected with purified wild-type (WT) or vaccinia virus recombinants (vHBs 1-5) at 30 plaque forming units (pfu)/cell or mock infected. At 2 hr, virus inoculum was replaced with 2.5 ml of Eagle medium containing 2.5% fetal bovine serum. Cells were harvested at 24 hr and separated from culture medium by centrifugation at 2,000 X g for 5 min. Cell pellets were suspended in 2.5 ml of phosphate buffered saline, frozen and thawed 3 times and sonicated. Equal portions of cell extracts and culture medium were tested for HBsAg by radioimmunoassay and for vaccinia virus by plaque assay in CV-1 cells. Culture medium and a cell extract prepared as above from a hepatoma cell line PLC/PRF/5 three days after confluency were tested in parallel for HBsAg.

TABLE 2

Production of antibodies against HBsAg

by rabbits vacciniated with recombinant vHBs4

RIA units/0.2 ml of serum

Days after vaccination	vHBs4	WT virus
5	92	8
6	135	—
7	352	—
8	>512	—
9	>512	—
10	>512	—
11	442	8

Undiluted serum, obtained from rabbits on the days indicated, was tested for antibody to HBsAg by a radioimmunoassay procedure (AUSAB, Abbot Laboratories). An HBsAg positive control human plasma supplied by Abbot Laboratories had a titer of 512 RIA units.

Deposition of Materials

One member of a group of plasmid vectors with chimeric HBsAg genes including pHBs2, pHBs4, and pHBs5, and one member of a group of vaccinia virus recombinants that expresses HBsAg including vHBs2, vHBs4, and vHBs5 were selected for deposition at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. Samples of E. coli HB101 transformed with pHBs4 were deposited on 12/1/82 and assigned ATTC No. 39250 . Samples of vHBs4 were deposited on 12/1/82 and assigned ATCC No. VR2055.

CLAIMS

1. Plasmid vectors containing a chimeric gene, composed of ~~poxvirus~~ vaccinia virus transcriptional regulatory sequences linked to the protein coding sequence of the HBsAg gene of hepatitis B virus, flanked by DNA from a non-essential region of the vaccinia virus genome.

2. Plasmid vectors according to Claim 1 wherein the ~~poxvirus~~ vaccinia virus transcriptional regulatory sequence is from the vaccinia virus thymidine kinase gene or from an early vaccinia virus gene encoding a polypeptide known as 7.5K.

3. Plasmid vectors according to Claim 1 wherein the flanking DNA consists of segments of the vaccinia virus thymidine kinase gene and neighboring DNA.

4. Plasmid vectors according to claim ~~one~~ 1 that are members of a group including pHBs2, pHBs4, and pHBs5.

5. Infectious ~~poxvirus~~ vaccinia virus recombinants that (a) contain a chimeric gene composed of ~~poxvirus~~ vaccinia transcriptional regulatory sequences linked to the protein coding sequences of the HBsAg gene of hepatitis B virus inserted into a non-essential region of the ~~poxvirus~~ vaccinia virus genome, (b) are produced by homologous recombination between infectious ~~poxvirus~~ vaccinia virus and plasmid vectors according to Claim 1, and (c) express the HBsAg upon infection of cells and elicit production of antibodies to HBsAg upon vaccination of animals.

6. Poxvirus recombinants according to Claim 5 that are members of a group including vHBs2, vHBs4 and vHBs5.

7. A method for producing ~~(a)~~ HBsAg by ~~infecting~~ cells with vaccinia virus recombinants according to Claim 5, and (b) antibodies to HBsAg in animals by vaccination with vaccinia virus recombinants according to claim 5.